RELATIVE SIZE DISCRIMINATION IN HONEYBEES (*APIS MELLIFERA*)

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By

Charis E. Dowell

Thesis Committee:

Dr. Lorey Takahashi, Chairperson

Dr. Scott Sinnett

Dr. Grayden Solman

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Abstract

Recent research found that honeybees are capable of learning complex types of learning such as oddity and same-different problems. Honeybees can learn these types of discrimination problems even when every trial differs from each other. However, there is a current lack of research showing the extent to which bees can solve relative size (larger versus smaller size) discrimination problems. Therefore, to address this issue, five experiments were conducted in free-flying honeybees trained in relative size discrimination problems. In Experiments 1 and 2, bees were trained in a size discrimination problem with a trial-unique procedure. The only difference between the two experiments was that the color within trials was the same in one experiment and different in the other (e.g., all green, versus green/orange). In Experiments 3 and 4, bees were trained in an oddity discrimination where relative size determined oddness. Again, the only difference between experiments was the color within trials. Lastly, in Experiment 5, bees were trained in a simultaneous same-different where relative size determined "sameness" and "difference." Results in all five experiments found that bees solved the relative size discrimination problems. In addition, in the last three experiments, honeybees were capable of solving relative size in other types of relational problems such as second order relational learning. These experiments add to a growing body of evidence that suggests honeybees are capable of solving complex learning problems thought to be a unique characteristic of vertebrates.

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Introduction

Vertebrate Background

A major focus of modern vertebrate research is to investigate the theories of animal learning using rat and pigeon subjects. The use of pigeons and rats was partly influenced by the apparatus created by Skinner (1938), which allowed these animals to be studied in a controlled setting. Further, rats and pigeons are highly motivated, easy to maintain and useful for cross comparisons among labs.

The data collected from rats offered information about fundamental learning principles, such as blocking, habituation, sensitization, peak shift, etc., that was also transferrable to human learning. However, questions about the evolutionary basis of learning started to arise. Bitterman was one of the first to take a comparative approach of the rat studies with other vertebrate species (Bitterman, 1975; Bitterman 1988; Bitterman 1996). The initial evolutionary assumption was that more complex learning was associated with more brain development. However, Bitterman (1988) found more similarities than differences in the performance of rats compared to other vertebrates. These findings strengthened the idea that the principles of learning are much the same across vertebrates. These similar results in many vertebrate species brought about an interest in using the same rat and pigeon approach to study invertebrate learning. One animal of interest was honeybees.

Honeybee Comparative Approach

Honeybees are an invertebrate of particular interest due to the research by Von Frisch (1950) in which he looked at the different sensory abilities that bees possess. He discovered that honeybees perceive colors very similar to that of humans. Interestingly, bees are not able to perceive the color red (long wavelengths), but can detect ultraviolet (short wavelengths). Not

only are honeybees capable of perceiving color but they can also detect vibration and touch (Kirchner, 1993; Nieh & Tautz, 2000; Rohrseitz & Tautz, 1999), as well as magnetic fields (Walker & Bitterman, 1985). In addition to these detection abilities, honeybees have many different motor functions including walking, flying, stinging and proboscis extension. Also, foraging honeybees are very motivated by sucrose and will not satiate since they always go back to the hive and regurgitate the sucrose into another bee, enabling the bee to fill up with sucrose over and over.

With associative principles, such as basic Pavlovian conditioning, laying the foundation of animal learning, much of the initial honeybee research focused on Pavlovian conditioning. (Bitterman, 1966). The easiest way to study associative learning is through the proboscis extension technique which involves restraining a bee to allow only minimum head and leg movement and the proboscis extension can then be studied. Based on the early research, associative principles of learning were suggested to account for many of the learning phenomena reported in honeybees (Dyer, Neumeyer, & Chittka, 2005; Giurfa, Eichmann, & Menzel, 1996; Gross, Pahl, Si, Zhu, Tautz, & Zhang, 2009; Howard, Avarguès-Weber, Garcia, Greentree, & Dyer, 2018). However, recent bee studies have begun to study complex forms of relational and abstract learning which are demonstrated to occur in vertebrates.

An ideal to study relational learning in bees is to use the free-flying technique, which involves training a bee to fly to a window to an artificial flower that contains a high sucrose solution. The bee must use color, odor, or some other factor to determine where to land on the artificial flower for the sucrose. After filling up on the sucrose, the bee flies back to the hive to replete and then may fly to and from the window. Common categories of relational learning or same-different discrimination include oddity, matching-to-sample, nonmatching-to-sample,

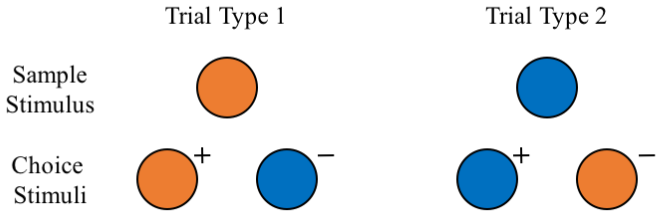
simultaneous same-different, and successive same-different (Table 1). In oddity, an animal is presented with a series of stimuli, and must select the odd stimulus to be rewarded. In a matching-to-sample procedure, an animal is first presented with a sample stimulus. After the presentation of the first stimulus, two subsequent stimuli are shown; one that is the same as the sample and another that is different from the sample. The goal is to pick the stimulus that matches the sample. Nonmatching-to-sample follows the same procedure but the goal is now to select the stimulus that does not match the sample. In a simultaneous same-different, an animal is presented with two pairs of stimuli. One pair is identical to each other and the other pair is different from each other. Not always, but typically, the two pairs are separated by some type of barrier. The goal can be either to always pick the same pair or the different pair. Finally, in successive same-different discrimination, a series of either identical or not identical stimuli are presented. Unlike simultaneous, where both the same and different pairs are presented at one time, the stimuli are presented separately. Hence, a series of identical stimuli would be presented first, one at a time, and then a different series of stimuli would be presented one at a time. After each set of same or different stimuli, the animal would need to press a corresponding key that is representational of a same or different set.

Table 1. Diagram of five common discriminations used to look at same/different concept learning.

Traditional Oddity

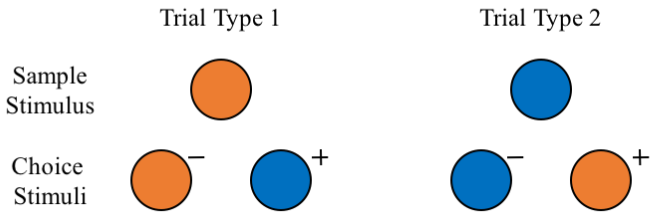
Single-Group Design
-Presented with three stimuli
-Rewarded for odd stimulus

Matching-to-sample



Single-Group Design
-Presented with a sample stimulus
-Simultaneously or successively presented with two choice stimuli
-Rewarded for stimulus that matches sample

Nonmatching-to-sample

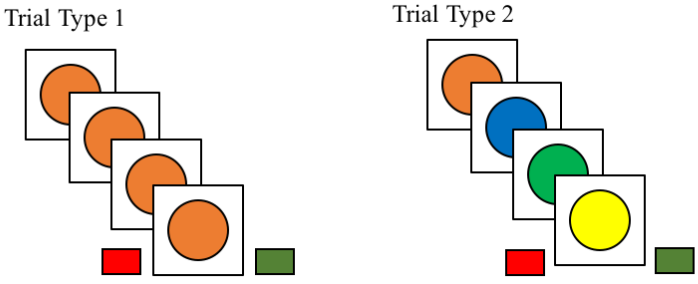


Single-Group Design
-Presented with a sample stimulus
-Simultaneously or successively presented with two choice stimuli
-Rewarded for stimulus that does not match the sample

Simultaneous Same-different

Two-Group Design
-Presented with two pairs of stimuli
-Group Same: Rewarded for same pair
-Group Different: Rewarded for different pair

Successive Same-different



Single-Group Design
-Presented with Trial Type 1: Select red key after viewing all stimuli successively
- Presented with Trial Type 2: Select green key after viewing all stimuli successively

Evidence of Relational Learning in Bees

Relational learning is of interest due to the fascination of what bees can learn. The goal of conducting matching/nonmatching-to-sample, oddity, and same-different was to extend the what bees can learn. Further, invertebrate research may offer novel insights into how learning may occur in a simple brain.

Matching-to-sample/Nonmatching-to-sample Experiments

Giurfa and colleagues (2001) examined the ability of bees to perceive sameness and difference using a delayed matching-to-sample procedure. Here, different patterns, colors, or odors were used in training and bees were rewarded with sucrose when selecting the stimulus that differed or matched the sample. Results showed that bees were successful in all training, reaching levels of 70% correct and were successful in transferring the relative concept of sameness and difference to novel stimuli.

Shishimi (2013) conducted a series experiments looking at both simultaneous matching-to-sample (MTS)/nonmatching-to-sample (NMTS) and delayed matching-to-sample/nonmatching-to-sample. Here, different color, patterns, and shapes were used in acquisition and bees were either rewarded for matching the sample or not matching the sample. The sample was either presented simultaneously or before (delayed) the stimulus pairs. Results showed that the bees were successful in the training reaching performance around 60% correct, indicating that bees learned sameness and difference.

Oddity Experiment

To address the oddity concept, Muszynski and Couvillon (2015) conducted a three-stimulus oddity problem with honeybees. Bees were presented with novel stimuli on every trial consisting of two-color patterned pinwheels in which the odd color of the pinwheels was rewarded. Bees learned the discrimination as evidenced by consistently selecting the odd stimulus about 50% of the time (chance = 33%) in only 15 trials. These results demonstrate that bees can solve a relational problem with a trial-unique procedure.

Simultaneous Same-different Experiments

Using bumblebees, Brown and Sayde's study (2013) showed the ability for bees to solve a simultaneous same-different problem. In their experiment, bumblebees were successful in selecting the correct door openings based on the 'sameness' and 'difference' of stimulus floor patterns. Further the bumblebees transferred this learning to novel stimuli. These findings support the idea that bees, in general, appear to solve same-different problems.

Lastly, Muszynski (2018) ran a series of experiments looking at simultaneous same-different problems. In her experiments, honeybees were trained in trial unique procedure with multi-colored pinwheels. Honeybees were rewarded with sucrose for either selecting the same colored pair or the different colored pair. The bees learned the discrimination in both conditions approximately 65% of the time. These findings further support that bees can learn simultaneous same-different problems. The results added to the literature indicating that bees truly can learn these more relational types of problems and do so well above chance.

Summary

Overall, several studies suggest that bees are capable of learning relational concepts using color, pattern, or odor stimuli. Further, these experiments cannot be explained with simple associative properties due to varying the stimuli in either size, shape, color, and/or pattern. However, other stimulus dimensions need to be explored and documented in honeybees to understand the extent of the relational learning concept in invertebrates. These other dimensions are important to study in order to understand what type of relational problems bees can solve in relation to vertebrates and potentially other invertebrates. One stimulus dimension of interest is size, which is relational. For example, whether a circle is considered "large" or "small" can only be determined when compared to another circle. Therefore, comparing size discrimination can be further studied as a relational problem. Of evolutionary interest, some studies have shown that bees may have evolved a predisposition to prefer large compared to small flowers that may support the hypothesis that foraging and selecting large flowers is more efficient in harvesting nectar for the hive than foraging for small flowers (Martin, 2004; Naug and Arathi, 2007). However, other studies found that selection of floral size preference may be a learning preference (Blarer, Keasar, & Shmida, 2002). These conflicting views notwithstanding, honeybees could still learn relative size.

Size Discrimination

Relative size is representative of the idea that determining size requires relational properties. Determining features such as "larger" and "smaller" require a comparison to other objects. Relative size discrimination is important for animals to survive. For example, which amount of food is larger may offer higher nutritional value. Further, avoiding which animals are

larger and more likely to be a predator is also necessary for daily survival. Therefore, relative size discrimination may be an important concept for animals to learn. However, compared to other forms of relational learning, much less is known about the concept of size discrimination especially in invertebrates.

Single-dimensional Approach on Vertebrates

The first experiment to investigate relative size was conducted by Bingham in 1913 on chickens. In his experiment, chicks were trained select the larger of two circles with the size of the circles varying over trials. His results found that the chicks picked the larger circle immediately, even when size was novel.

Like Bingham's study, the idea of simply presenting an animal with larger or smaller stimuli on the same continuum from training is considered a single-dimensional approach. This approach was used to study fish (Mark & Maxwell, 1969), elephants (Nissani, Hoefler-Nissani, Tin Lay, & Wan Htun, 2005), a sea lion (Schustermann & Krieger, 1986), penguins (Manabe, Murata, Kawashima, Asahina, & Okutsu, 2009), crows (Moll & Nieder, 2014) and dogs (Keep, Zulch & Wilkinson, 2018). In all studies, except for the elephants and the fish, the animals were successful in solving relative size discrimination on a single-dimensional level. These findings suggest that when presenting animals with novel sizes of the same stimulus type, vertebrates are capable of learning relative size.

Multi-dimensional Approach on Vertebrates

A number of experiments have used the multi-dimensional approach that involves novel stimuli that vary in more than just size from the training stimuli. These experiments include a parrot (Pepperberg & Brezinsky, 1991), horses (Hanggi, 2003), a dolphin (Murayama, Usui,

Takedam, Kato, & Maejima, 2012), and dogs (Byosiere, Feng, Chouinard, Howell, & Bennett, 2017). Phenomenally, all of these studies have been successful.

Pepperberg and Brezinsky (1991) conducted a study on a single African gray parrot by the name of Alex. In training, Alex had to verbally state whether a variety of different objects were larger or smaller than other objects. Further, Alex identified which object was the middle size out of three. Results indicated the parrot was successful in selecting the correct stimulus 80% of the time. These results were not altogether surprising since Alex previously showed similar success in other types of problems.

Hanggi (2003) investigated the horses' abilities to learn on the basis of relative size. Here, different shapes and colors were presented in either a two-dimensional or three-dimensional formatting. The horses were either rewarded with food for selecting the larger or the smaller of two stimuli. Even with novel shapes, colors, and dimensions, horses successfully selected the rewarded size 80% of the time.

Murayama and colleagues (2012) examined size discrimination in a dolphin. The dolphin was rewarded by selecting the larger of two stimuli. Over the training trials, the stimuli differed in shape from each other. The dolphin learned to select the larger of two stimuli 90% of the time even when the shapes were novel.

A recent study involved training dogs with different sized shapes and rewarding them for selecting the larger of the two stimuli (with one exception where a dog was rewarded for the smaller of two stimuli). Dogs were able to successfully learn the discrimination 80% of the time for most of the shapes presented. However, the dogs could not transfer the discrimination to triangles, vertical bars, or horizontal bars. Although these finding suggest that dogs can learn

relative size, they do not appear capable of transferring the relation over to various symbolic objects (Byosiere, et al., 2017).

Taken together, these results suggest that vertebrates are generally capable of learning relative size and when factors change in relational situations. These results clearly demonstrate that vertebrates are able to learn and adapt to the many changing relational factors on a day-to-day basis in the environment.

Experiments on Invertebrates

In contrast to the many relative size studies conducted in vertebrates, only four invertebrate studies were conducted. This includes three studies with bees (Avarguès-Weber, Amaro, Metzler & Dyer, 2014; Howard, Avarguès-Weber, Garcia, & Dyer, 2017; Wiegermann, Wiegermann, MacNeal, & Gafford, 2000) and one with ants (Beugnon & Macquart, 2016).

In the giant ant study (Beugnon & Macquart, 2016), ants were trained in a 6-chambered maze to use the relative size (larger versus smaller) of vertical bars to determine which direction to go in the maze. Their results found that the ants learned to discriminate the vertical bars to navigate the maze around 60% correct. While the performance was not optimal, it was still better than chance and hinted at size discrimination.

Wiegermann and colleagues (2000) worked on bumblebees to assess relative height discrimination. The bees were presented with different colored flowers varying in height. In pre-training, bees were all presented with the same-sized flower. In training, some bees were given the control (same flower from pre-training), whereas some bees were rewarded for selecting the smaller of two artificial flowers. Lastly, in the test trial, all bees were presented with the pre-training flower and a smaller flower. Their findings indicated that bees in the control selected the

pre-training flower in the test trial while those rewarded for smaller flowers instead selected the smaller flower in the test trial. These results, therefore, suggest that the bees learned something about relative height.

Lastly, two experiments were conducted with honeybees. In both experiments, bees were presented with a series of yellow squares/diamonds varying in size. The bees were rewarded with sucrose for either selecting the larger or the smaller set of stimuli. In later training, the bees were presented with novel star shapes with varying colors. In all conditions, the bees successfully selected the rewarded stimulus regardless of what size was rewarded (Avarguès-Weber, Amaro, Metzler & Dyer, 2014; Howard, Avarguès-Weber, Garcia, & Dyer, 2017). Although both experiments suggest that honeybees are able to learn relative size, there are some concerns about the bees learning on the basis of associative principles since the stimuli are not unique on every trial. Also, the methodological structure of both experiments was not fully addressed to determine the role the experimental methodology may play in the different results.

Current Novel Experiments

Although many vertebrate species appear successful in assessing relative size discrimination, the extent to which this complex cognitive capability is fully shared in invertebrates remains to be determined. Therefore, the current series of experiments were designed to investigate whether the invertebrate honeybee is capable of exhibiting relative size discrimination.

The purpose of Experiment 1 examined whether or not honeybees could learn the relative size concept with novel stimuli on every trial. That is, honeybees were evaluated on their ability

to successfully select either the larger or smaller stimulus differing in pattern, color, or shape within trials.

Experiment 2 extended to results of Experiment 1 by incorporating relative size factors that varied in a simulated everyday situation of color. Although Experiment 1 examines relative size, Experiment 2 was necessary to determine if this type of problems extends to varying factors such as color. Here, honeybees were again required to select the larger or smaller pattern and shape, but colors differed within stimulus pairs.

To increase the complexity of relative size discrimination and to expand what scientists know about bee learning, Experiment 3 evaluated whether honeybees learn a second-order relational concept. Honeybees were exposed to a three-stimulus oddity problem in which identical pattern, color, and shape were tested within each trial and rewarded with sucrose only when selecting the odd size. Honeybee success in solving this second-order oddity problem would demonstrate, for the first time, that invertebrates are capable of acquiring a more complex second-order form of relational learning.

Experiment 4 determined whether honeybee learning in Experiment 3 could further occur in the three-stimulus oddity problem when pattern and similar shape were identical within each trial but color now differed for each stimulus choice. Honeybees were rewarded with sucrose when selecting the correct odd size.

Experiment 5 introduced relative size discrimination in a same-different problem. The goal was to see if honeybees could further learn a relative size discrimination within another relation learning problem. Honeybees were now trained on a simultaneous same-different problem with size as the distinguishing feature of sameness and difference. Bees were rewarded for selecting either the same stimulus pair or the different stimulus pair in which identical

pattern, color and similar shape were used within trial. Positive results will add to an emerging body of work demonstrating the invertebrates are as capable of solving second-order relational problems as in vertebrates.

Experiment 1: Relative Size – Same Color

Evidence suggest that bees have an innate preference for larger than smaller flowers to maximize the collection of nectar (Martin, 2004; Naug & Arathi, 2007). However, other studies have found that floral size preference is learned regardless of size (Blarer, Keasar, & Shmida, 2002). These differing findings suggest that while bees may prefer larger flowers in certain naturalistic instances, it appears that bees learn which flowers (regardless of size) provide the most nectar. Therefore, this experiment determined whether bees discriminate relative size in a trial-unique procedure, where the stimulus pairs were novel on every trial. The only differing factor among each stimulus pair would be the size of the stimuli. Two groups were trained, one rewarded with sucrose on the larger of the two stimuli (Group Large) and one rewarded on the smaller of the two stimuli (Group Small). The research will also assess whether honeybees have an innate preference to constantly select larger stimuli over smaller size stimuli with equivalent amounts of sucrose.

Method

Subjects

Twenty-four experimentally naïve honeybees (*Apis mellifera*) were from hives in the back of the Békésy Laboratory of Neurobiology located at the University of Hawai'i at Mānoa. They were collected from feeders, adjacent to the hives, containing 10-20 percent

sucrose solution. Twelve bees were assigned to Group Large and 12 bees to Group Small. Each bee was individually trained over a few hours in a single session.

Apparatus and Stimuli

The apparatus used for pre-training and training was a wooden enclosure attached to the wall of the laboratory (Figure 1). The dimensions were 61cm wide, 61cm high and 61cm deep, and the outside of the enclosure had two wooden doors that could be opened to allow bees access to the enclosure from outside. The experimenter was inside the laboratory and could monitor the bee through a pair of transparent Plexiglas doors which also prevented the bee from entering the laboratory.

Figure 1. Apparatus used for all experiments.

All stimuli were selected from a pool of stimuli used in previous experiments. Two stimuli were used for pre-training and 12 pairs of stimuli used for training. All stimuli are shown

in the sample sequence illustrated in Figure 2 One pre-training stimulus and 10 of the pairs were fabricated with colored vinyl plastics (blue, yellow, green, orange) cut to create a variety of shapes, including circles, triangles, squares, and rectangles (trials P1, 1, 2, 4, 5, 7, 8, 9, 10, 11, and 12 in Figure 2). Six pairs were created by gluing the colored shapes onto the top of Petri dishes painted with gray primer, thus producing different patterns (trials 4, 5, 8, 9, 10, and 12 in Figure 2). The second pre-training stimulus (P2) was a Petri dish painted with gray primer. The last two pairs of stimuli were created from Plexiglas chips covered with spray enamel (trials 3 and 6 in Figure 2). The pair of stimuli on trial 3 was white Plexiglas sprayed with black enamel to create a striped appearance and the pair of stimuli on trial 6 was sprayed completely with colored enamel.

Pre-training

A foraging honeybee was individually selected from a feeder containing 10-20 percent sucrose solution. The bee was captured in a matchbox from the feeder and brought to the experimental enclosure located inside of the laboratory. The first pre-training stimulus (P1 in Figure 2) was placed in the center of the wooden enclosure with a 100-µl drop of 50% sucrose solution in the top center of the stimulus. Holding the matchbox directly over the sucrose, the experimenter opened the matchbox until the bee's proboscis touched the sucrose and began to drink. Once sucrose drinking commenced, the matchbox was fully opened to allow the bee to settle on the stimulus and marked with a dot of nail polish on its abdomen for identification. The bee eventually flew out of the enclosure and back to the hive to unload the sucrose. Bees were expected to return to the enclosure instead of the feeder because the pre-training stimulus contained 50% sucrose which is preferred over the 10-20 % sucrose at the feeders. If within ten

minutes the marked bee returned to the apparatus, a second pre-training stimulus (P2 in Figure 2) was presented which also featured a 100-µl drop of 50% sucrose solution. However, if the bee did not return, the experimenter checked the feeder and either recaptured the marked bee or collected new bees from the feeder to place on the pre-training stimulus. Pre-training ended when an individual bee returned on its own to the second pre-training stimulus and drank sucrose to repletion.

Training

After exposure to the second pre-training stimulus, the bee was tested during training by presenting it to a pair of stimuli in the center of the enclosure set 10.2 cm apart edge-to-edge. One stimulus was larger than the other, but both consisted of the same color and pattern, and generally the same shape. One of the stimuli contained a 100-µl drop of 50% sucrose (reward), and the other contained a 100-µl drop of 10% stevia solution (punishment). Stevia is aversive to honeybees and cannot be discriminated from the sucrose solution except by taste. Twelve bees were rewarded for choosing the larger of the two stimuli (Group Large) and another 12 bees were rewarded for choosing the smaller stimulus (Group Small). If the bee first landed on the rewarded stimulus, the response was recorded as correct. However, if the bee first landed or touched the stevia solution on the non-rewarded stimulus, the response was recorded as an error. When this happened, the bee typically withdrew its proboscis and left the stimulus. A correction procedure was used to allow the bee to continue to choose until it selected the rewarded stimulus, drank, flew back to the hive, and returned to the enclosure for the next training trial. Although only the initial choice was analyzed, the experimenter recorded any repetitive errors on each trial, which were relatively rare.

Each bee underwent a trial-unique training procedure consisting of 12 training trials with a different pair of stimuli on each trial. There were 16 different stimulus sequences used for the training of the 24 bees, with either one or two bees trained with each sequence. The position of the rewarded stimulus was balanced across training trials with the left position of the pair rewarded on half the trials and the right position rewarded on the other half. The rewarded position (left or right) was never repeated for more than two trials in a row. Since there were common shapes, patterns, and colors among the stimulus pairs, the stimulus sequences were created so that the same color, shape or pattern never occurred on two trials in a row. Furthermore, care was taken to ensure that each stimulus pair occurred in the early trials in some sequences and in the later trials in others. The sequences were essentially the same for the bees in Group Large and Group Small. A sample sequence is shown in Figure 2.

Each bee completed the 12 training trials in one session. The time between training trials, the inter-trial interval, was measured by the experimenter with a stopwatch. The start of the interval was when the bee lifted off the rewarded stimulus to return to the hive, and the end of the interval was when the bee returned to the enclosure and made its initial choice on the next trial. The session for each bee lasted approximately 2-3 hours, after which the bee was captured in a matchbox and removed from the experimental window.

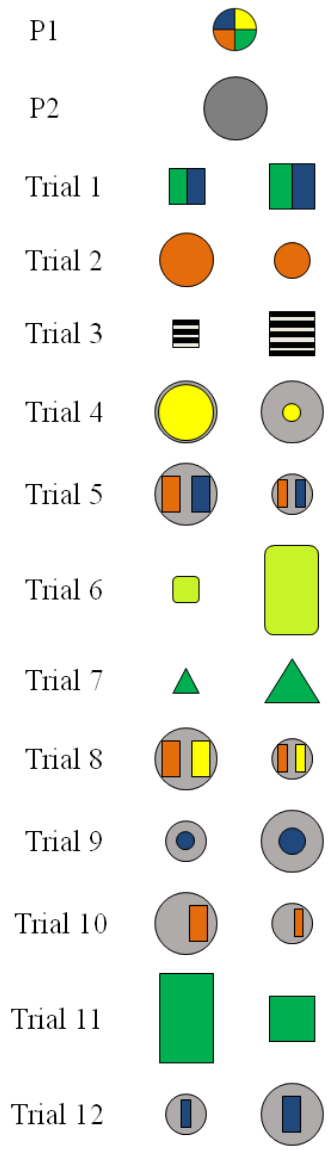


Figure 2. Sample sequence for Experiment 1. P1 stands for the first pre-training stimulus and P2 stands for the second pre-training stimulus.

Results

In Group Large, the overall mean proportion of correct choice was .71 with a range of .58-.83 for the individual bees (Figure 3). The mean was significantly greater than chance [t (11) = 8.55, $p < .001$, SEM = .024]. In Group Small, the overall mean proportion of correct choice

was .74 with a range of .67- .92 for the individual bees (Figure 3). The mean was also significantly greater than chance [t (11) = 11.9, p < .001, SEM = .021]. An analysis of variance of the proportion correct for the first block of six trials and the last block of six trials found no significant Group effect [F (1, 22) = .833, p = .371], no significant Block effect [F (1, 22) = 2.137, p = .158] and no Group by Block interaction [F (1, 22) = .107, p = .747]. The analyses indicate the bees had no significant preference for larger or smaller stimuli. The absence of significant block effects suggests that performance in the first half of training did not differ from performance in the last half of training. Inspection of Figure 3 shows the bees learning the discrimination after two or three trials.

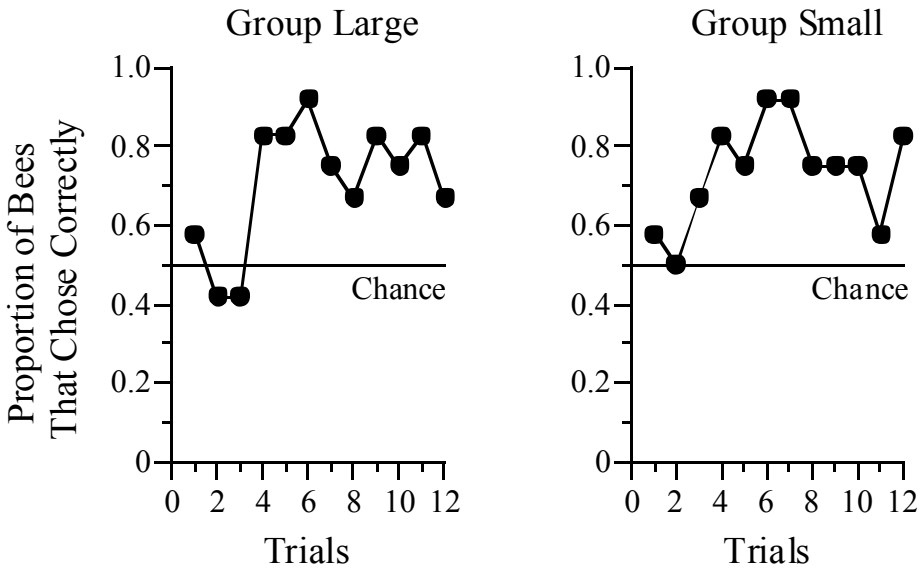


Figure 3. The proportion of bees that chose correctly in both Group Large (left panel) and Group Small (right panel). The horizontal line at .50 indicates chance performance.

Position preference is the tendency to land on the stimulus in one position (left or right). To assess whether bees have a position preference, the initial choices on the left and right stimuli for each trial were calculated for all the bees. The Group Large mean proportion of left/right

choices was .55/.45. A t-test of the proportion of left choices against the expected value of .5 (chance) was not significant [t (11) = 1.259, p = .23]. The Group Small mean proportion of left/right choices was .54/.46, which again did not differ significantly from chance [t (11) = .898, p = .39]. These findings suggest that the bees had no position preferences that interfered with the discrimination of large versus small.

In difficult discriminations, animals will sometimes simply follow the position of the reward from trial to trial. In Group Large, the mean proportion of choices of the position rewarded on the preceding trial was .46. The mean was not significantly different from .5, the chance value expected if the bees were not following position [t (11) = -1.136, p = .28]. Group Small, the mean proportion of choices of the position rewarded on the preceding trial was .41. The mean again was not significantly different from chance [t (11) = -1.188, p = .087]. Therefore, the bees were not showing a position reward, which could be a significant source of error in the results.

Discussion

This experiment demonstrated that honeybees are capable of learning the concept of size discrimination and there was no difference in performance between them in the discrimination. Furthermore, there was no evidence of an innate preference for large or for small since both groups did equally well. Clearly, the bees can solve a relative size problem with novel stimulus pairs on every trial.

Nonetheless, the problems appear to be difficult for both groups as evidenced by the terminal performance which reached a proportion of correct choice of about .75 in the last half of the discrimination training. For comparison, in simple color discriminations, honeybees reach an

asymptote of about .90 or higher. The trial-to-trial variability in performance also suggests that the problems are difficult. The difficulty of the problems could simply be that relational problems are more difficult than simple discrimination problems, especially when using a trial unique procedure. Nonetheless, it seemed prudent to look for any systematic sources of error in the bees' choices. However, the data did not show any indication of a position preference or reward following pattern, and did not interfere with the bees' learning of the discrimination. Additional training may be required to reach a high, consistent asymptote.

While these findings show that bees learn relative size, the data do not indicate how they are learning the discrimination. The relative size difference could be conceptualized as relative height, width, circumference, area, and/or amount of color. One likely possibility is relative amount of color because color is very salient to honeybees (Von Frisch, 1950).

Experiment 2: Relative Size – Different Color

Experiment 2 extends the results of Experiment 1 and assesses the role of different color in relative size discrimination by varying the color of stimulus pairs within each trial. Varying the color within trials was to see if performance altered with more variability. In everyday situation, honeybees are presented with many different colored flowers, so changing the colors in each trial should not alter performance. As in Experiment 1, bees were required to discriminate relative size with novel stimulus pairs but stimuli not only differed in size, but also in color in each trial. Two groups were trained, one rewarded on the larger of the two stimuli (Group Large) and one rewarded on the smaller of the two stimuli (Group Small).

Method

Subjects

Twenty-four experimentally naïve honeybees (*Apis mellifera*) were from hives in the back of the Békésy Laboratory of Neurobiology located at the University of Hawai'i at Mānoa. They were collected from feeders, adjacent to the hives, containing 10-20 percent sucrose solution. Twelve bees were assigned to Group Large and 12 bees to Group Small. Each bee was individually trained over a few hours in a single session.

Apparatus and Stimuli

The apparatus used in Experiment 1 was also used for this experiment (Figure 1). All of the stimuli used in Experiment 1 were also used in Experiment 2 with a slight variation. The two pre-training stimuli stayed the same. For 11 pairs of stimuli, the only difference was that one of stimuli in each of the pairs was switched with a different color. The stimuli in trial 8 from Experiment 1 (Figure 2) were removed and replaced with a pair of vinyl twelve-point stars. All of the stimuli are shown in the sample sequence illustrated in Figure 4. Materials used and the process to create them were the exact same as from Experiment 1.

Pre-training

Pre-training was the same as Experiment 1.

Training

Training was the same as the first experiment, except that one stimulus in each pair was a different color (Figure 4).

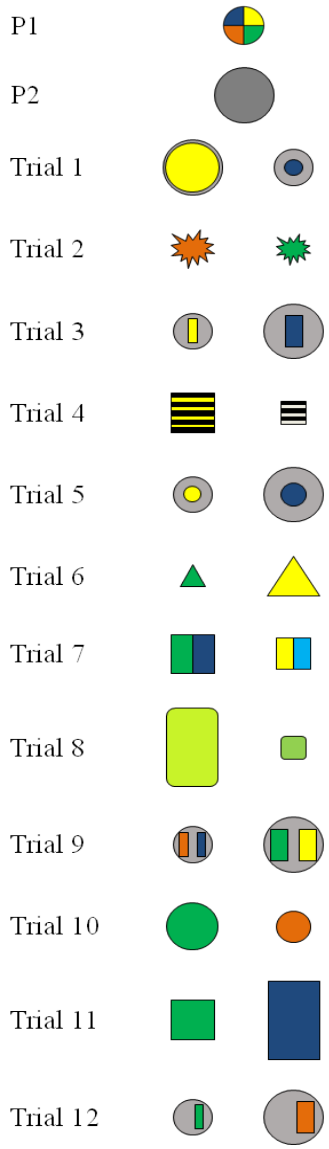


Figure 4. Sample sequence for Experiment 2. P1 stands for the first pre-training stimulus and P2 stands for the second pre-training stimulus. Note that the color within each trial is now different between the stimulus pairs.

Results

In Group Large, the overall mean proportion of correct choices was .78 with a range of .67 - .83 for the individual bees (Figure 5). The mean was significantly greater than chance [t (11) = 13.21, $p < .001$, SEM = .0174]. In Group Small, the overall mean proportion of correct choice was .73 with a range of .58 - .92 for the individual bees (Figure 5). The mean was also significantly great than chance [t (11) = 9.36, $p < .001$, SEM = .0296]. An analysis of variance of the proportion correct for the first block of six trials and last block of size trials found no significant Group effect [F (1, 22) = 2.62, p = .120], a significant Block effect [F (1, 22) = 9.44, p = .006] and no Group by Block interaction [F (1, 22) = .518, p = .479]. These finding indicated the bee's performance was better in the last half of the trials compared to the first half, for both groups, but performance between the group did not differ significantly.

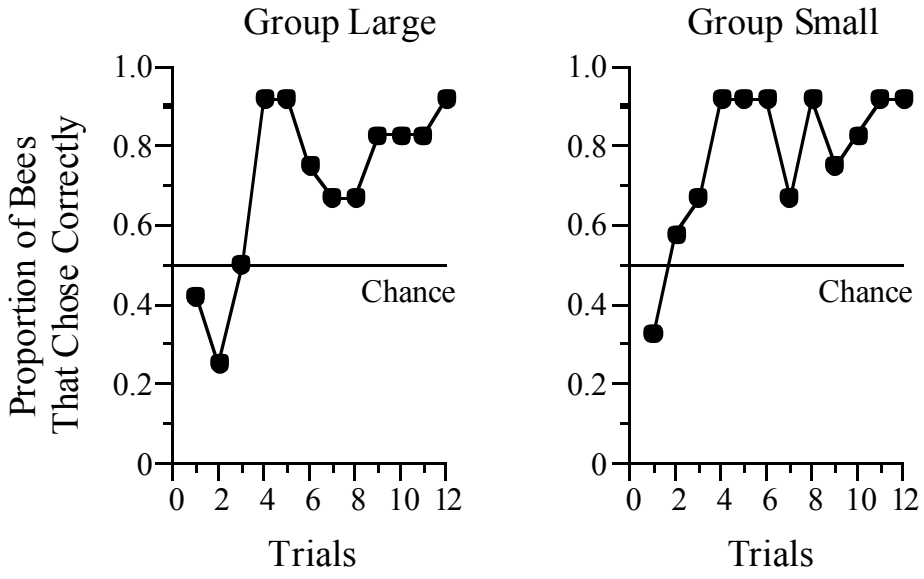


Figure 5. The proportion of bees that chose correctly in both Group Large (left panel) and Group Small (right panel). The horizontal line at .5 indicates chance performance.

The data were analyzed to determine if the bees have a position preference and whether bees prefer to select the position rewarded on the immediately preceding trial. For position preference, the initial choices on the left and right stimuli for each trial were calculated for all of the bees. For the bees in Group Large, the mean proportion of left/right choices was .51/.49. A t-test of the proportion of left choices against the expected value of .5 (chance) was not significant [t (11) = .44 $p > .05$]. For the bees in Group Small, the mean proportion of left/right choices was .51/.49, which again did not differ significantly from chance [t (11) = .37, $p > .05$]. These findings suggest that bees tested in either the Large or Small group showed no position preferences that significantly interfered with the discrimination of large versus small.

In Group Large the mean proportion of choices of the position rewarded on the preceding trial was .35. The mean was not significantly different from .5, the chance value expected if the bees were not following position [t (11) = -3.46, $p < .05$]. For the bees in Group Small, the mean proportion of choices of the position rewarded on the preceding trial was .36. The mean again was not significantly different from chance [t (11) = -5.03, $p < .05$]. Therefore, position rewarded on the preceding trial does not appear to be a significant source of error in the results.

To compare the results from Experiment 1 and Experiment 2, the last eight bees of each experiment (Four bees from Group Large and four bees from Group Small in each experiment) were run intermixed in order to run an analysis of all the bees. The performance of bees in Experiment 1 and Experiment 2 are plotted in Figure 6 as the proportion of bees that chose correctly in each training trial. While slight variability is seen in the graphs, an overall ANOVA found no difference between the two groups [F (1, 14) = 1.8, p = .187]. There was also no difference between the small and large subgroups [F (1, 14) = 2.77, p = .103], and no interaction among all conditions [F (1, 14) = .14, p = .71].

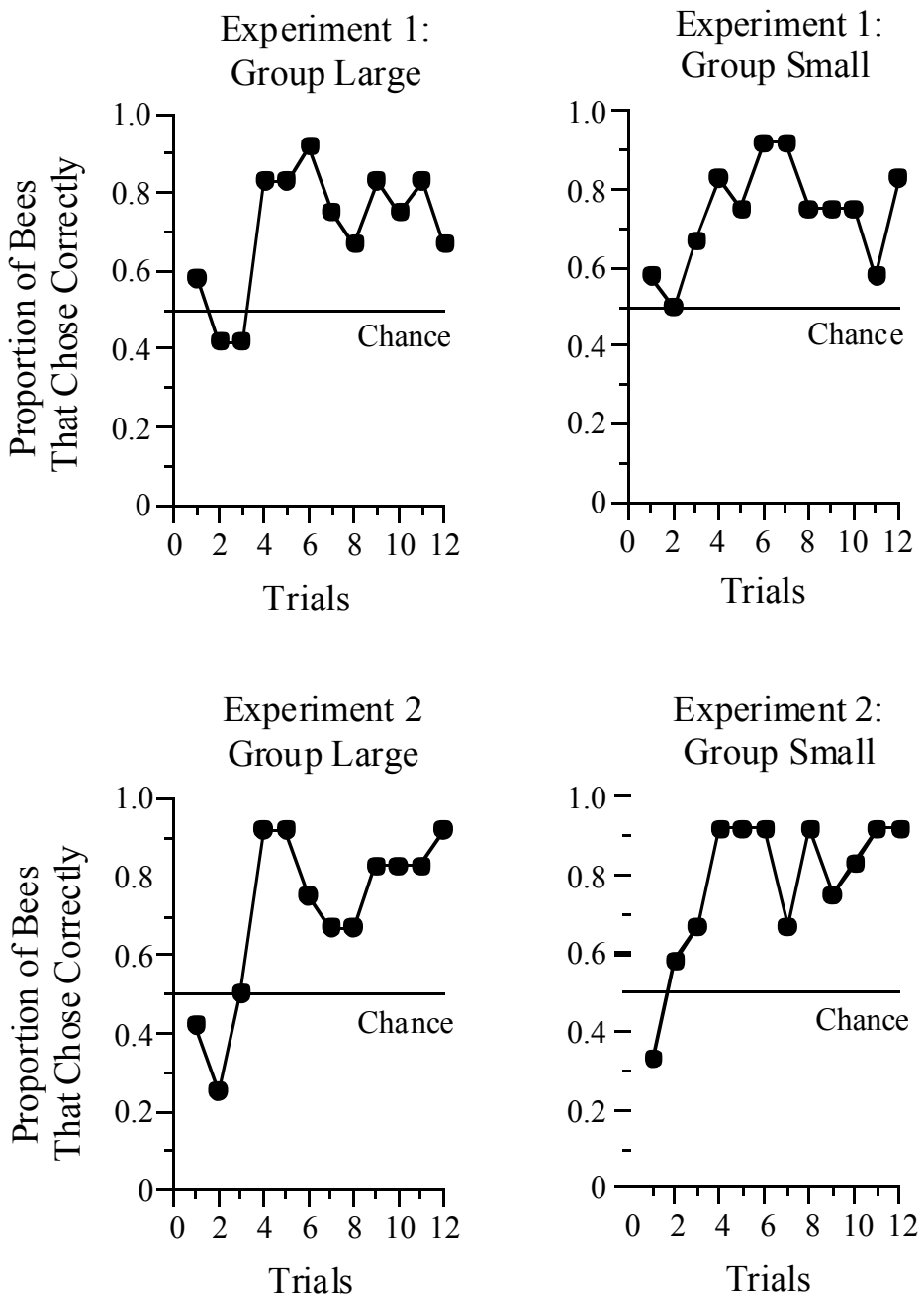


Figure 6. For Experiment 1 (top row) and 2 (bottom row), the proportion of the bees that chose correctly on each trial when the rewarded stimulus was either small or large.

Discussion

The results of Experiment 2 indicate that honeybees learned the size discrimination as in Experiment 1 without a preference for large or small size. In addition, honeybees learned the discriminating relationship even with varying colors within trials. Clearly, a bee can solve relative size problems even when other factors, such as color, vary.

Experiment 3: Oddity and Relative Size – Same Color

Evidence hints at vertebrates' ability to learn oddity and relative size (Davis, Leary, Stevens, & Thompson, 1967; Thomas & Frost, 1983). This type of learning encompasses a larger type of discrimination known as second-order relational learning. Second-order relational problems require that an animal learn two different relational properties to solve the problem (e.g. how the stimuli differ in size and what stimulus is odd in relation to the others). Currently, no invertebrate studies exist that look at second-order relational learning, including oddity and size. Therefore, this experiment determined whether bees discriminate relative size in a three-stimulus oddity problem, where the stimulus pairs were novel on every trial.

Method

Subjects

Twelve experimentally naïve honeybees (*Apis mellifera*) were from hives in the back of the Békésy Laboratory of Neurobiology located at the University of Hawai'i at Mānoa. They were collected from feeders, adjacent to the hives, containing 10-20 percent sucrose solution.

Apparatus and Stimuli

The same apparatus was used from Experiment 1 and 2 (Figure 1). Stimuli were pulled from the previous experiments. Both pre-training stimuli stayed the same (trials P1 and P2 in Figure 7). Thirteen sets of stimuli were created from stimuli used in Experiment 1 and 2 (trials 1, 2, 4, 5, 6, 7, 8, 9, 10, 12, 13, 17, and 18 in Figure 7). Five additional sets were added. Three sets were fabricated with colored vinyl plastics (blue, orange, green) which were cut to create a few different shapes, including squares, rectangles, and triangles. Two sets of stimuli were vinyl (trials 3 and 15 in Figure 7), while one set was created by gluing the colored vinyl onto a Petri dish with gray primer (trial 16 in Figure 7). The last two sets of stimuli were created from colored Plexiglas chips that were cut into squares (trials 11 and 14 in Figure 7).

Pre-training

Pre-training was the same as Experiment 1 and 2, with one variation. Instead of placing the two pre-training stimuli in the middle of the enclosure, the first pre-training stimulus was placed in the left, middle, or right side of the enclosure, with the second pre-training stimulus being placed in one of the other two locations. The placement of the two pre-training stimuli was randomized across bees. This was done to prevent the bees from having a side preference in the training trials.

Training

After exposure to the second pre-training stimulus, the bee was tested during training by presenting it with three stimuli in the center of the enclosure that were each set 2.5 cm apart edge-to-edge. Two of the three stimuli were the same (nonodd) and were either larger or smaller

than the third stimulus (odd). All stimuli were the same color, and pattern, and generally the same shape. The odd stimulus contained a 100 µl drop of 50% sucrose (reward), and the two nonodd stimuli contained a 100-µl drop of 10% stevia solution (punishment). Besides this experiment being an oddity discrimination, the rest of the training procedure followed that of Experiment 1 and 2.

Each bee underwent 18 training trials and one extinction trial. There were 12 different stimulus sequences used for the training of the 12 bees, with one bee trained on each sequence. The position of the odd stimulus was balanced across training trials with the left position of the set rewarded on a third of the trials, the middle position rewarded on a third of the trials, and the right position rewarded on a third of the trials. The odd position (left, middle, or right) was never repeated sequentially. Since there were common shapes, patterns, and colors among the stimulus sets, the stimulus sequences were created so that the same color, shape or pattern never occurred on two trials in a row. Furthermore, care was taken to ensure that each stimulus set occurred in the early trials in some sequences and in the later trials in others. A sample sequence is shown in Figure 7. Each bee completed the 18 training trials in one session. The session for training each bee lasted approximately 2-4 hours, and lead immediately into an extinction trial.

Extinction

Immediately following the 18 training trials, the bee was given with a "19^{th}" trial. In extinction, the bee was presented with a novel set of stimuli, one odd, and two nonodd. However, instead of a 100-µl drop of sucrose or stevia solution, all three stimuli contained a 100-µl drop of tap water. While there is no indication that a bee can visually discriminate the sucrose versus stevia, this extinction trial was given to confirm that the bee persisted in choosing the odd when

all three of the stimuli contained the same unacceptable-tasting liquid. After returning from the last training trial, the bee was presented with three stimuli with water. All landings to all three stimuli were recorded for 10 minutes. After the 10 minutes, the bee was captured in a matchbox and frozen.

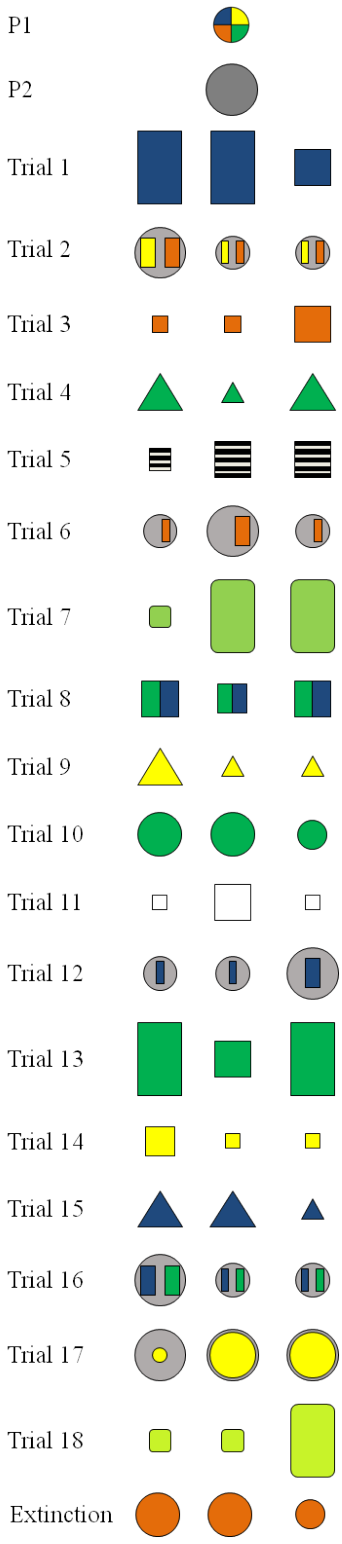


Figure 7. Sample sequence for Experiment 3. P1 stands for the first pre-training stimulus and P2 stands for the second pre-training stimulus. In each trial, the bee must not only determine the

relative size of the stimuli (what is larger or smaller from each other), but must also determine which stimulus is odd compared to the two nonodds.

Results

Acquisition Results

The overall mean proportion of correct choice was .62 with a range of .33-.72 for the individual bees (Figure 8). The mean was significantly greater than chance [t (11) = 9.51, p < .001, SEM = .031]. Performance for when the odd stimulus was small and odd stimulus was large were also analyzed separately for the individual bee. The mean for when the odd was large was .58 and was significantly greater than chance [t (11) = 5.75, p < .001, SEM = .043] and the mean for when the odd was small was .65 and was also significantly greater than chance [t (11) = 8.15, p < .001, SEM = .039]. Furthermore, there was no difference between performance when the odd was large or small [F (1, 11) = 1.6, p = .232].

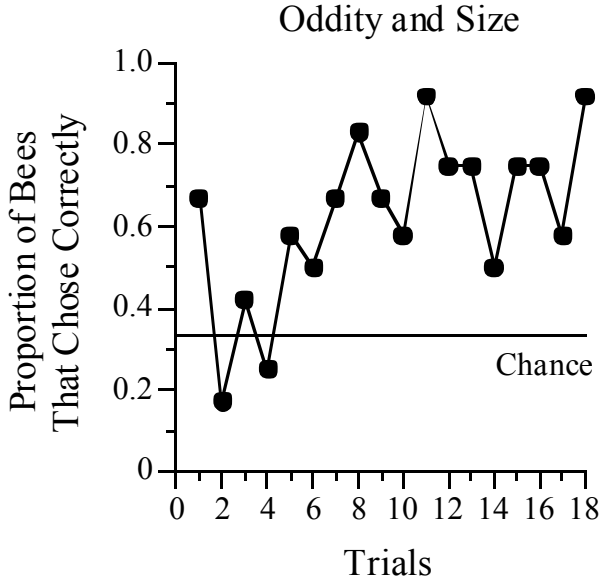


Figure 8. The proportion of bees that chose correctly. The horizontal line as .33 indicates chance performance.

The data were analyzed to determine if the bees have a position preference. For position preference, the initial choices on the left, middle and right stimuli for each trial were calculated for all of the bees. A chi-square goodness of fit test for the three positions was not significant [$X^2(2) = 2.028$, $p = .363$]. This finding suggests the bees had no position preferences that interfered with the discrimination of odd versus nonodd.

Extinction Results

The mean total number of landings for the odd stimulus was 22.4 while the mean total number of landings for both nonodd was 11.5 and 14.9 (Figure 9). A paired sample t-test found a significant difference between landings on the odd compared to landings on the nonodd [t (11) = 5.042, $p < .001$].

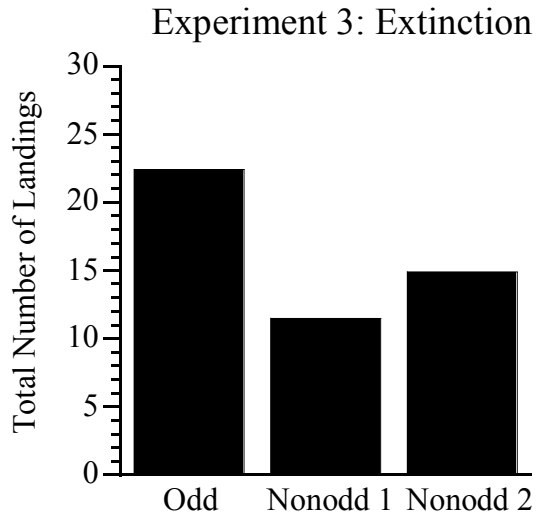


Figure 9. Extinction plotted as the mean total number of landings.

Discussion

The results of Experiment 3 indicate that honeybees learned the oddity and size discrimination. Therefore, honeybees appear to be the first invertebrate to solve a second-order relational problem due to their ability to simultaneously discriminate size and oddity to solve the discrimination. Since the results from Experiment 1 and 2 showed no difference in performance, it seemed plausible that varying color in a size discrimination oddity problem should not alter performance either. Therefore, performance in Experiment 4 should yield similar results as found in Experiment 3.

Experiment 4: Oddity and Relative Size – Different Color

Experiment 4 extends the results of Experiment 3 and assesses the role of color in an oddity and size discrimination by varying the color of the stimulus pairs within trial. As in Experiment 3, bees were required to discriminate oddity and relative size with novel stimulus sets, but the odd stimulus not only differed in size, but all stimuli varied in color in each trial.

Methods

Subjects

Twelve experimentally naïve honeybees (*Apis mellifera*) were from hives in the back of the Békésy Laboratory of Neurobiology located at the University of Hawai'i at Mānoa. They were collected from feeders, adjacent to the hives, containing 10-20 percent sucrose solution.

Apparatus and Stimuli

The same apparatus was used from the previous experiments (Figure 1). All stimuli used in Experiment 3 were also used in this current experiment with a slight variation. The two pre-training stimuli were the same, but two of the three stimuli in each set were switched with a different color. All stimuli are shown in a sample sequence illustrated in Figure 10. Material used and the process to create them were the same as from Experiment 3.

Pre-training

Pre-training was the same as Experiment 3.

Training

Training was the same as the third Experiment, except that all three of the stimuli in each set were a different color (Figure 10).

Extinction

Extinction was the same as Experiment 3 except now all three stimuli were a different color (Figure 10).

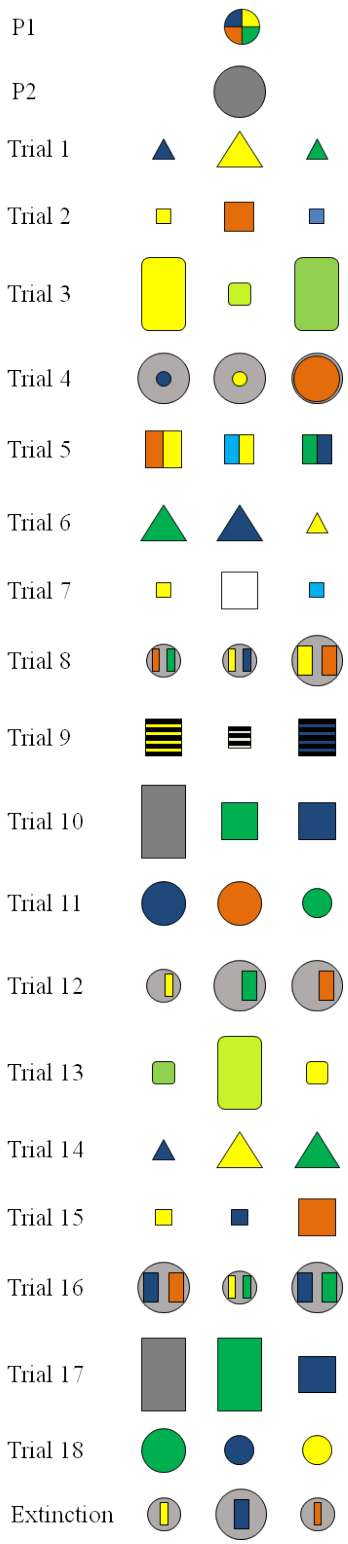


Figure 10. Sample sequences for Experiment 4. P1 stands for the first pre-training stimulus and P2 stands for the second pre-training stimulus.

Results

Acquisition Results

The overall mean proportion of correct choice was conducted to see the total performance of the bees over the 18 trials. The overall mean was .59 with a range of .33-.77 for the individual bees (Figure 11). The mean was significantly greater than chance [t (11) = 9.172, $p < .001$, SEM = .031]. Performance for when the odd stimulus was small and performance for when the odd stimulus was large were also analyzed separately for the individual bee. The mean for when the odd was large was .50 and was significantly greater than chance [t (11) = 8.147, $p < .001$] and the mean for when the odd was small was .69 and was also significantly greater than chance [t (11) = 7.916, $p < .001$]. Furthermore, there was no difference between performance when the odd was large or small [F (11, 1) = 12.791, $p = .004$].

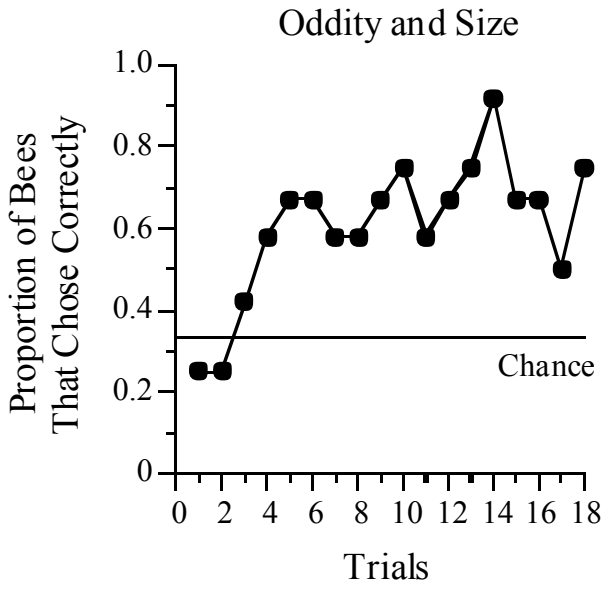


Figure 11. The proportion of bees that chose correctly. The horizontal line as .33 indicates chance performance.

The data were analyzed to determine if the bees have a position preference. For position preference, the initial choices on the left, middle and right stimuli for each trial were calculated for all of the bees. A chi-square goodness of fit test for the three positions was not significant [$X^2(2) = .778$, $p = .678$]. This finding suggests the bees had no position preferences that interfered with the discrimination of odd versus nonodd.

To compare the results from Experiment 3 and Experiment 4, the 12 bees in each experiment were analyzed together. The performance of bees in Experiment 3 and Experiment 4 are plotted in Figure 12 as the proportion of bees that chose correctly in each training trial. While slight variability is seen in the graphs, an overall ANOVA found no difference between the two groups [$F(1, 22) = .218$, $p = .645$].

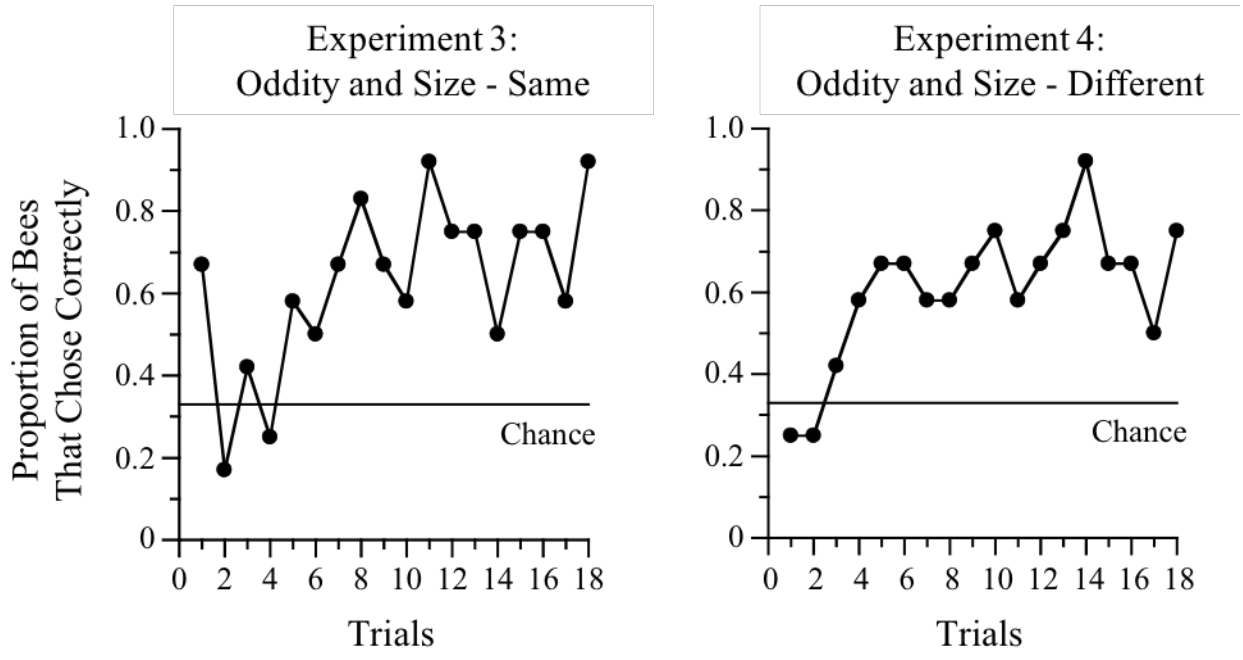


Figure 12. For Experiment 3 (left panel) and 4 (right panel), the proportion of the bees that chose correctly on each trial. Experiment 3 and Experiment 4 are titled the same, except for the

indication of "same" and "different" to represent whether the color is the same or different within a trial.

Extinction Results

The mean total number of landings for the odd stimulus was 19.42 while the mean total number of landings for both nonodd was 12.7 and 11.5 (Figure 13). A paired sample t-test found a significant difference between landings on the odd compared to landings on the nonodd [t (11) = 4.738, p = .001].

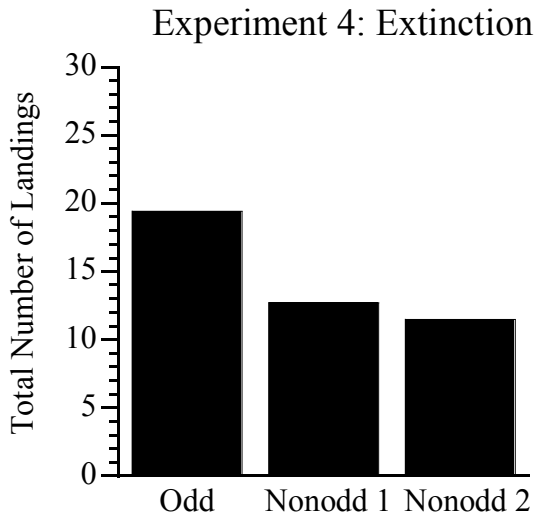


Figure 13. Extinction plotted as the mean total number of landings.

Discussion

The results of this experiment suggest that bees learned the oddity and size discrimination as in Experiment 3. In addition, bees learned these relationships even with color variation in each

trial. Clearly, honeybees can solve oddity and relative size even when factors differ in color. Furthermore, the results strengthen that bees can learn a second-order relation.

Experiment Five: Simultaneous Same-Different and Relative Size – Same Color

The final experiment extends the results of Experiment 3 and 4 and tests second-order relational learning, but with relative size and simultaneous same-different. In this experiment, the bees not only need to determine which pairs are the same and different, but they must use the relative size of the pairs to solve the problem. Two groups were trained, one rewarded for selecting the pair with the same sizes (Group Same) and one rewarded for selecting the pair with different sizes (Group Different).

Methods

Subjects

Eight experimentally naïve honeybees were from hives in the back of the Békésy Laboratory of Neurobiology located at the University of Hawai'i at Mānoa. They were collected from feeders, adjacent to the hives, containing 10-20 percent sucrose solution. Four bees were assigned to Group Same and four bees to Group Different. Each bee was individually trained over a few hours in a single session.

Apparatus and Stimuli

The apparatus used in the last four experiments was also used for this experiment (Figure 1). The stimuli were pulled from the previous experiments (Figure 14).

Pre-training

The pre-training stimuli was the same as those used the previous experiments. The only variation is that one of the pre-training stimuli was placed on the right side of the enclosure and the other on the left side in pseudo-random order. This was to prevent an initial side preference in training.

Training

After exposure to the second pre-training stimulus, the bee was tested during training by presenting it two pairs of stimuli in the center of the enclosure. Three stimuli were the same color, shape, pattern and size, while the last stimulus was the same color, shape, and pattern, but differed in size. In the middle of the enclosure a wooden blocker 25 cm in length, .5 cm in width, and 3 cm in height was used to separate the two pairs. The pairs were separated 3 cm away from the blocker and within each pair, the stimuli were 1 cm apart. One of the pairs has a 100 µl drop of 50% sucrose on each of the stimulus (reward) and the other pair had a 100-µl drop of 10% stevia solution on each of the stimulus (punishment). Four of the bees were rewarded for choosing the "same" pair (Group Same), and four bees were rewarded for choosing the "different" pair (Group Different). The rest of the training followed that of the previous experiments.

In this experiment, each bee underwent 16 training trials. There were 4 different stimulus sequences used for the 8 bees, with two bees trained on each sequence (one in Group Same and one in Group Different). The position of the pairs was balanced across training trials and the rewarded pair was never repeated for more than two trials in a row. Since there were common shapes, patterns, and colors among the stimulus pairs, the stimulus sequences were created so

that the same color, shape or pattern never occurred on two trials in a row. Furthermore, across the sequences care was taken to ensure that each stimulus pairs occurred in the early trials in some sequences and in the later trials in others. Each bee completed the 16 training trials in one session (Figure 14).

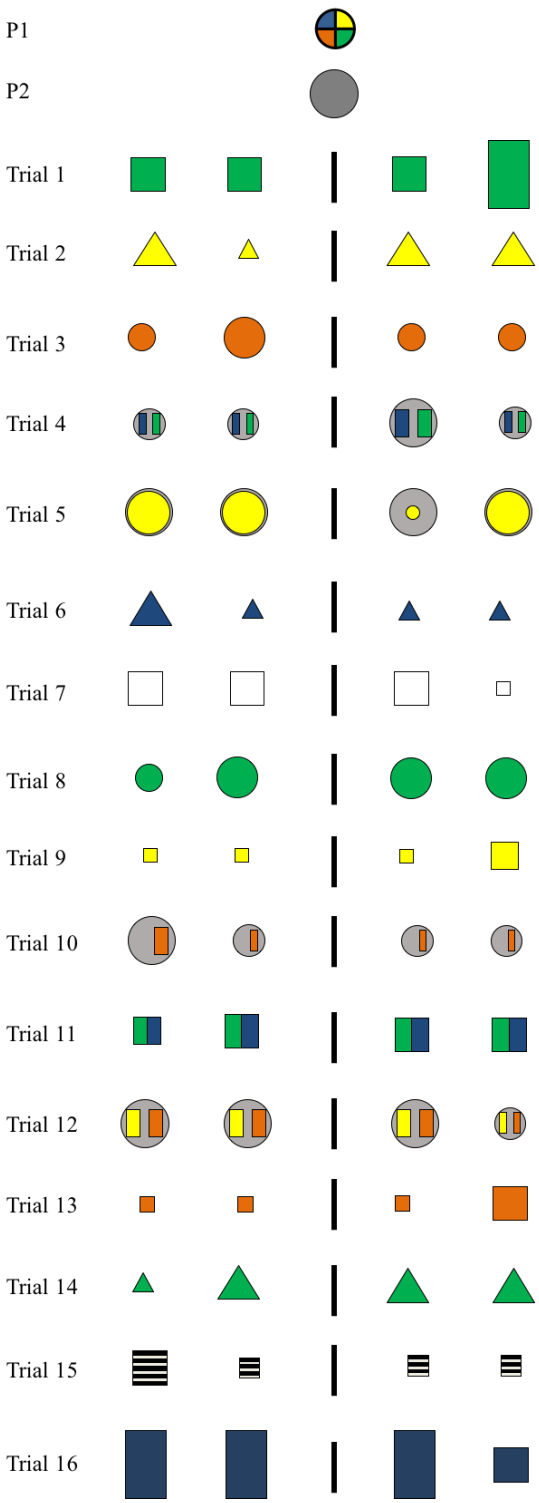


Figure 14. Sample sequences for Experiment 5. P1 stands for the first pre-training stimulus and P2 stands for the second pre-training stimulus. The black lines represent the wooden barrier.

Results

In Group Same, the overall mean proportion of correct choice was .69 with a range of .56-.75 for the individual bees (Figure 15). The mean was significantly greater than chance [t (3) = 4.19, p = .03, SEM = .045]. For the bees in Group Different, the overall mean proportion of correct choice was .65 with a range of .63- .69 for the individual bees (Figure 15). The mean was also significantly great than chance [t (3) = 9.67, p < .001, SEM = .015]. An analysis of variance of the proportion correct for the first block of eight trials and the last block of eight trials found no significant Group effect [F (1, 3) = .391, p = .537], no significant Block effect [F (1, 3) = 1.09, p = .306] and no Group by Block interaction [F (1, 3) = .043, p = .836]. The findings indicate no difference between the groups. The absence of ~~a~~ significant block effects suggests the performance in the first half of training did not differ from performance in the last half of training.

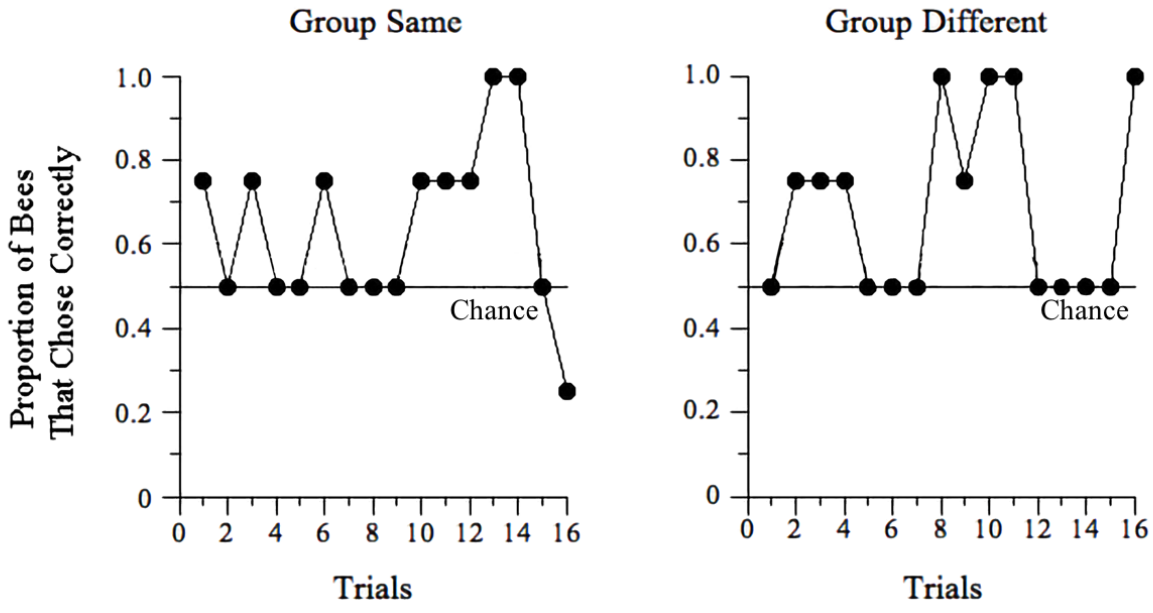


Figure 15. The results for Experiment 5 plotted as the proportion of bees that chose correctly in both Group Same (left panel) and Group Different (right panel). The horizontal line as .50 indicates chance performance.

The data were analyzed to determine if the bees have a position preference and whether bees prefer to select the position rewarded on the immediately preceding trial. In Group Same, the mean proportion of left/right choices was .46/.54. A t-test of the proportion of left choices against the expected value of .5 (chance) was not significant [t (3) = -.403, p = .71]. In Group Different, the mean proportion of left/right choices was .49/.51, which again did not differ significantly from chance [t (3) = -.234, p = .83]. These findings suggest that bees had no position preferences that interfered with the discrimination of same versus different.

In Group Same the mean proportion of choices of the position rewarded on the preceding trial was .43. The mean was not significantly different from .5, the chance value expected if the bees were not following position [t (3) = -2.61, p = .08]. For the bees in Group Different, the mean proportion of choices of the position rewarded on the preceding trial was .47. The mean again was not significantly different from chance [t (3) = -.454, p = .681]. Therefore, position rewarded on the preceding trial does not appear to be a significant source of error in the results.

Discussion

This final experiment demonstrated that bees are capable of learning same-different discrimination with relative size. Furthermore, there was no evidence of a preference for choosing the same or different pair. These findings indicate that bees can learn a second-order relational discrimination.

General Discussion

As a whole, the current experiments show that honeybees can learn relative size discrimination with novel stimuli on each trial. While Avarguès-Weber and colleagues (2014) first established that honeybees could learn the concept of relative size, these studies indicate that they can succeed at the discrimination even when each trial is unique. By utilizing a trial-unique procedure, the experiments ruled out that the bees simply responded to the features of a specific stimulus (Wright et al., 1988).

The first two experiments addressed whether bees could solve a relative size discrimination utilizing a trial-unique procedure. The differing factor between Experiment 1 and Experiment 2 was to see whether changing the color within each trial would alter performance. Further, these experiments were to acknowledge if bees might have a preference for flower size. The results yielded that bees appear to learn a relative size problem regardless of whether the colors differ within the trials or not. Also, these two experiments added to the existing literature that flower choice for bees appears to be a learned preference (Blarer et al., 2002).

These results also indicate that bees' performance in a discrimination task is similar to the vertebrate literature but differences in testing procedures may underlie some differences in the testing performance of bees and vertebrates. That is, vertebrate performance reaches approximately 80% correct in relative size discrimination whereas the bees performance reached around 70% correct (Hanggi, 2003; Murayama, Usui, Takedam, Kato, & Maejima, 2012; Pepperberg & Brezinsky, 1991). These differences in performance maybe due to the testing duration, which extended over a period of several days in vertebrates and only a few hours in the honeybee.

In addition, Experiments 3, 4 and 5 suggest that honeybees can also learn second-order relational problems. These types of discrimination require an animal to learn two different relational concepts to solve a problem. In Experiment 3 and Experiment 4, the bees were required to solve an oddity and relative size problem, with the only difference being whether the colors were the same or varied within trials. In these experiments, solving the relationship problem involved determining which stimuli differ in size as well as which stimulus is odd. In Experiment 5, bees were trained to solve a simultaneous same-different problem. This final second order relational learning experiment required the bees to determine both the relative size of the stimuli and whether the stimuli are the same or different. These three studies found that honeybees are able to solve these types of problems and are likely the first invertebrate to solve them. The ability of bees to learn these problems play a significant role in suggesting that more complex forms of learning are found not only in vertebrates, but also in honeybees (an invertebrate). These findings are quite surprising since the history of animal learning has suggested that relational learning like that of Experiments 3, 4, and 5 were only possible in language-trained chimps (Premack, 1983).

Future directions should attempt to address the same-different and relative size discrimination with varying colors within trials. Also, adding more subjects to Experiment 5 and then comparing it to the color varied experiment would be fascinating. It would be interesting to see if the performance is not altered as in Experiment 2 and 4. Further, the current experiments did not account for the specific size differences within each trial. Therefore, future researchers might consider addressing the performance of bees when the size is varied strategically to see when the bees are able to discriminate the difference in size. Lastly, conducting a relative size discrimination between two-dimensional and three-dimensional stimuli might yield some

fascinating novel results about their perception when dimensions change, especially since it did not appear to have an influence on horses (Hanggi, 2003). The novel research, therefore, expands what is now known about the learning abilities of honeybees and gives scientists a better understanding of what can be learned from such a tiny invertebrate.

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